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(54) Title: SPECIFIC IMPENDING OVULATION INDICATOR

(57) Abstract

Method for detecting impending ovulation in the human female by testing means employable by the average person. The test method involves contating vaginal fluid samples with a bibulous mat containing an antibody against estrogen-induced peroxidase. The mat is then washed and tested for peroxidase.

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Description

Specific Impending Ovulation Indicator

Technical Field

This invention relates to methods of detecting impending ovulation in human females, including methods which are sufficiently simple so that a woman can carry out the test on herself without the aid of a physician.

Background Art

While prior methods of fertility or ovulation testing have been proposed, prior tests have one or more undesirable aspects. For example, in the prior art the method of utilizing thermometry (the basal body temperature method) provides information on fertility, but this test merely indicates that ovulation has already occurred and does not detect impending ovulation.

The prior ovulation detection methods involving an examination of cervical mucus for its flow properties, saline content, glucose content and the like, are also deficient in that they do not easily lend themselves to self-examination by the woman and require sampling of the posterior portion of the vagina. Similarly, microscopic examination of vaginal cells for staining characteristics and morphology require expensive apparatus and involve techniques which are usually beyond the skill of the average woman. Estrogen analysis of blood and urine is likewise complicated and difficult to carry out.

Means for peroxidase testing have been known since 1898. Such means have been used for over 75 years for the detection of blood, including commercial articles sold, for example, by Smith Kline and French Laboratories and by Miles Laboratories, for the detection of occult blood in urine and feces. Despite the long history

of the peroxidase test it has not been previously used to determine the surge in estrogen-induced peroxidase in vaginal fluid for the detection of impending ovulation. This may be due to the fact that pathological conditions or injuries resulting in blood being present in the vagina contribute to a false positive indication for estrogen-induced peroxidase. It is known that hemoglobin and its degradation products exhibit a peroxidase-like reaction and, indeed, this property is utilized in the occult blood test for urine and feces.

Disclosure of Invention

In the approximately two to three days prior to ovulation in the menstrual cycle of normal human females there is a surge in the amount of estrogen-induced peroxidase (EIP) in the vaginal fluid. A simple form of the invention contemplates the woman taking a sample of vaginal fluid with, for example, a moistened cotton swab and contacting the swab with, for example, a bibulous mat containing an antibody against EIP. After contact of the swab with the mat, the mat is washed with water to remove interfering substances which do not combine with the antibody. An EIP-antibody combination possesses peroxidase activity, hence a positive peroxidase test on the aforesaid bibulous mat which had been in contact with a vaginal fluid sample and washed, indicates that EIP was present in the vaginal fluid. A negative test for peroxidase indicates the absence of EIP. If EIP is found, it indicates impending ovulation. An advantage of the present invention is that a specific antibody to EIP is used and this antibody combines only with EIP and not with the interfering substances. Chromogenic substrates of peroxidase and a hydroperoxide are used as a test for peroxidase.

Best Mode For Carrying Out The Invention

A simple method for the detection of impending ovulation in women, as in the present invention, is of the highest importance to the human race and is of paramount interest to such prestigious organizations as the Population Council, the Ford Foundation, the National Institutes of Health and the World Health Organization. With the present invention a woman can, by herself, determine her impending fertile time. There is extensive clinical evidence that for women the fertile time commences within 12 to 72 hours of ovulation (C. Tietze Fertility and Sterility, Vol. 11, p. 485, 1960). By abstaining from coitus or by otherwise protecting herself from insemination during the fertile time, a woman can avoid pregnancy. Thus with the aid of the present invention, which enables a woman to determine her fertile time, this form of birth control could, if practiced widely, substantially reduce the rate of world population growth. Using the present invention one may practice birth control without interference with the normal female hormonal function, such as occurs with the contraceptive pill which is objectionable to certain segments of the world population on religious grounds, as well as to others on medical grounds due to the possible serious side effects. A woman practicing abstinence during the fertile period as determined by the present invention may avoid the need for contraceptive devices, such as the intrauterine device, for birth control which are considered undesirable to some.

The present invention may also be an aid to couples who wish to have a child, but have failed because of, for example, incorrect timing of coitus. Thus it may be seen that because the present invention can be a valuable aid in family planning, it serves an important humanitarian purpose.

According to the present invention the woman moistens a cotton swab with vaginal fluid and then brings it into contact with a bibulous mat containing an antibody against EIP. The mat is then washed and tested for the presence of peroxidase with a hydroperoxide and a chromogenic substrate of peroxidase.

The hydroperoxide may be hydrogen peroxide or a hydroperoxide generating system, such as the inorganic peroxides, sodium peroxide, barium peroxide, strontium peroxide, sodium perborate, and the like, or organic hydroperoxides, such as methyl hydroperoxide or ethyl hydroperoxide. Many compounds, for example, sodium peroxide, barium peroxide, strontium peroxide, sodium perborate, and the bis (1-hydroxyalkyl) peroxides generate hydrogen peroxide when moistened. Enzymatic reactions such as the action in air of L-amino oxidase on L-amino acids also generate hydrogen peroxide. The word hydroperoxide as used herein and in the claims is meant to include all of the compounds and types of compounds of this type including the hydroperoxide generating compounds and enzyme systems which generate hydrogen peroxide.

A substance which becomes colored in the presence of peroxidase and a hydroperoxide is designated herein as a chromogenic substrate of peroxidase or as a chromogen. Chromogenic peroxidase substrates or chromogens which may be employed in the present invention include the following substances:

1) Monoamines, such as aniline and its derivatives, orthotoluidine, para-toluidine, etc.;
2) Diamines, such as ortho-phenylenediamine, N,N dimethylpara-phenylenediamine, N,N diethyl-phenylenediamine, benzidine, 3,3',5,5', tetramethyl benzidine, dianisidine, o-tolidine, etc.;

3) Phenols, such as phenol per se, thymol, ortho, meta and para-cresols, alpha-napthol, p,p-dihydroxybiphenyl, phloroglucinol and guaiacol;

4) Aromatic acids, such as salicylic, pyrocatechuic and gallic acids;

5) Leucodyes, such as leucomalachite green (to produce malachite green) and leucophenolphthalein (desirably employed in an alkaline medium);

6) Colored dyes, such as 2,6 dichlorophenol indophenol;

7) Various biological substances, such as epinephine, the flavones, tyrosine, dihydrophenylalanine (producing an orange-reddish color) and tryptophane. Other substances such as gum guaiac, guaiaconic acid, Nadi reagent (producing a bluish color), bilirubin (producing a greenish color), iodides (which produce a brown color and, if starch is present, produce a deep blue color which is much stronger than iodide alone).

Some of the substances may be most effectively used in combination rather than individually. For example, Nadi reagent is such a mixture, namely naphthol and p-phenylenediamine, which gives a better final color than the individual components. Another example is a mixture of 4-amino antipyrine and 1,7-dihydroxynaphthaline.

Many of the chromogens, notably 3,3',5,5' tetramethyl benzidine, orthotolidine and p,p'-biphenol give a more intense color if halogen ions, such as iodide and bromide ions, or if halogenoid ions, such as thiocyanate and selenocyanate ions, are present.

One of the preferred chromogens of the invention comprises 3,3',5,5' tetramethyl benzidine and potassium

thiocyanate; this mixture yields an intense blue color in a positive test. Another preferred chromogen is a mixture of p,p'-biphenol and potassium thiocyanate.

Substrates which change their fluorescence in the presence of a peroxidase and hydrogen peroxide include a loss of fluorescence of scopoletin or a production of fluorescence with, for example, dichlorofluorescin or homovanillic acid. Chemiluminescence is produced in the presence of peroxidases and hydrogen peroxide for the following typical substances, Luminol, zinc tetra phenylporphyrine and the like.

Antibodies against human vaginal fluid EIP may be prepared by injecting rabbits with human vaginal fluid EIP purified according to the procedures described by E. R. DeSombre and C.R. Lytlle in Cancer Research, Volume 38, November 1978, pp. 4086-4099; but, instead of using rat mammary tumor extract, the said human vaginal fluid sample is used. The injections into rabbits take place at regular intervals together with Freund's complete adjuvant in a manner well known to those skilled in immunology. After immunization to EIP has been achieved, antisera are collected. The antisera may be treated by methods well known to immunologist to obtain globulin samples which are enriched in EIP antibodies. The term antibody as used herein and in the claims denotes pure antibody against EIP, purified antibody against EIP, solutions comprising antibody against EIP, mixtures comprising antibody against EIP, antisera against EIP and also includes antibody against the apoenzyme of EIP. Not only will antibodies against EIP function in the invention, but also antibodies against the apoenzyme of EIP will function in this invention and should be understood to be included herein and in the claims.

Instead of injecting rabbits in the aforestated procedure for preparing the antibody to EIP, the technique involving lymphocyte hybridomas may be employed. Lymphocyte hybridoma techniques are described in the book entitled Lymphocyte Hybridomas published by Springer Verlag Berlin, Heidelberg: New York, 1978. The technique for making monoclonal antibodies by the technique of lymphocyte hybridomas is described in detail in the chapter by M. M. Trucco, J. W. Stocker, and R. Ceppelini in the aforesaid book, where in place of human lymphoblastoid cells employed by the aforesaid workers, purified EIP from human vaginal fluid samples or the apoenzyme of it may be used. Monoclonal antibodies against different antigenic determinants in EIP or its apoenzyme may be mixed to effect a precipitating antibody. Either monoclonal or mixed monoclonal antibodies may be employed in the present invention.

Some animals may produce species specific EIP which may be similar enough so that the antibodies to it may cross-react with human vaginal fluid EIP. In such cases animal vaginal fluid EIP may be used to produce antibodies which may be used in place of the human antibody to EIP. Tissue cultures of human estrogen-sensitive tissue, such as human endometrium and the like, may produce the apoenzyme of EIP, in which case EIP apoenzyme from such tissue cultures may be used as a source of apoenzyme which may be used in the present invention.

The apoenzyme of human EIP may be produced by the chemical synthesis of DNA and recombinant DNA methods well known to those skilled in the art. Expression in E. Coli of chemically synthesized genes for human EIP or its apoenzyme may be carried out as described by D. V. Goeddel et al. in Proc. Nat. Acad. Sci. USA, Vol. 76,

January 1979, pp. 106-110 where instead of human insulin one uses the aforesaid purified human EIP or its apoenzyme.

A preferred embodiment of the present invention comprises antibody to EIP attached to a solid support. There are many methods well known to those skilled in the art of affixing an antibody to a bibulous mat or to the surface of other solid support materials such as wood, plastic, glass, ceramic and the like. Said solid support may be in the form of a film, spheres, beads, tubes, ion exchange materials (such as glass beads comprising arylamino groups or comprising carboxyl groups such as produced by Corning Glass Works), filter paper loaded with ion exchange resins (such as produced by Whatman Paper Company), or paper loaded with Duolite ion exchange resin (the resin being made by Diamong Shamrock Company). With ion exchange resins the antibody is adsorbed but the antibody is not covalently linked. Antibody may be covalently linked to solid supports which comprise aryl amino groups or which comprise carboxyl groups by methods well known in the art. For example, the aryl amino groups on solid supports may be diazotized with nitrous acid (sodium nitrite and freshly added hydrochloric acid), washed with water and then treated with a solution comprising the antibody in sodium bicarbonate solution. Antibody may be coupled to carboxyl groups on solid supports by means of the carbodiimide reaction.

Example 1

Vaginal samples are taken daily starting on day 5 of a woman's menstrual cycle, where day 1 is taken as the day when menstruation began. Such vaginal samples can be obtained using a standard six inch cotton-tipped

swab ("Puritan", Hardwood Products Co.). The cotton end can be moistened with water and rolled gently on the wall of the anterior vagina.

The swab, while still moist, is then contacted with a paper to which was affixed an antibody to estrogen-induced peroxidase. After contact for 10 to 30 minutes, the paper was washed in water. The still moist paper containing the antibody is pressed against starch-iodide test paper and the test paper is moistened with 0.01% hydrogen peroxide. A strong blue color is regarded as a positive test and weak or no coloration is regarded as a negative test.

For best results it is preferable to start on day 5 or day 6 for the daily routine. The first positive test may be taken to indicate that ovulation is impending and will follow within two or three days.

In place of starch-iodide test paper, filter paper impregnated with 3,3',5,5' tetramethylbenzidine and potassium thiocyanate, impregnated with p,p' biphenol and sodium thiocyanate, or guaiac, or orthotolidine or other chromogenic substrates may be used. The "Hemoccult" test of Smith Kline and French Laboratories, which according to the manufacturer consists of paper impregnated with orthotolidine, may also be used.

Example 2

In place of the cotton-tipped swab of Example 1, the vaginal fluid sample is obtained by directly contacting the vaginal wall with a piece of moist filter paper to which an antibody to estrogen-induced peroxidase was attached. The filter paper was affixed toward the end of a plastic strip about 0.012 inches thick, 0.20 inches wide and 8 inches long and made of cellulose acetate.

The paper is removed from the vagina after 20 minutes and was then washed with water. While still moist the paper is contacted with the occult blood detector part of the commercially available "Hemicombistix" (Ames Company), which according to the manufacturer comprises orthotolidine and cumene hydroperoxide. A positive test (i.e., strong blue coloration) indicates impending ovulation.

In place of the occult blood detection part of "Hemicombistix", the occult blood peroxidase detector manufactured by Boehringer Company may be used, which detector, according to the manufacturer, contains orthotolidine and dimethyl dihydroperoxy hexane.

In place of the above-mentioned peroxidase detectors, starch-iodide paper containing dry sodium perborate may be used.

Example 3

In place of the plastic strip in Example 2 in which the antibody to estrogen-induced peroxidase is toward one end and a separate occult blood detector is provided on another strip, a strip was made with both the antibody and the blood detector as parts of the same strip, the antibody being at one end and the detector at the other end. The contact between the two was achieved by folding the strip at its middle and holding the ends in contact after the antibody end had been contacted with the vagina and had been washed.

Example 4

A swab similar to that of Example 1 and containing vaginal fluid is dipped into a 1% solution of sodium chloride contained in a test tube. Affixed to the inner wall of the test tube. Affixed to the inner wall of the test tube is an antibody to estrogen-induced peroxidase. After 30 minutes the swab is

removed from the test tube and the test tube is rinsed with water. Into the test tube is poured a solution containing 0.001% 3,3',5,5' tetramethylbenzidine to which had been freshly added 0.005% final concentration of hydrogen peroxide. A blue color is a positive indication of estrogen-induced peroxidase in the vaginal sample.

Example 5

A swab similar to that of Example 1 and containing vaginal fluid can be dipped into a solution containing 0.1 milligram per milliliter of rabbit antisera against estrogen-induced peroxidase. After 30 minutes activated charcoal is added. It is occasionally stirred, and the suspension is filtered. To the filtrate is added 0.001% final concentration of 3,3',5,5' tetramethylbenzidine and 0.1% final concentration potassium iodide, to which a 0.005% final concentration of hydrogen peroxide is freshly added. A blue color is a positive indication of estrogen-induced peroxidase in the vaginal sample.

Example 6

A swab similar to that of Example 1 and containing vaginal fluid can be dipped into a solution containing 0.01 milligram per milliliter of mixed clonal antibody to estrogen-induced peroxidase as described herein. The mixture is centrifuged and the supernatent is allowed to stand for 12 hours. The system is centrifuged again, the supernatent discarded, and the pellet was suspended with stirring into a 0.1% sodium chloride solution. After the system is again centrifuged, the pellet is contacted with starch-iodide paper and moistened with a 0.0005% hydrogen peroxide. A blue coloration is a positive indication of estrogen-induced peroxidase in the vaginal sample.

Claims

1. An indicator for detecting impending ovulation comprising a substrate which includes an antibody against estrogen-induced peroxidase in vaginal fluid samples and means for association therewith to detect estrogen-induced peroxidase immunoadsorbed by said antibody.
2. An indicator according to claim 1, wherein said means includes a chromogenic substrate of peroxidase.
3. An indicator according to claim 1, wherein said means includes a hydroperoxide.
4. An indicator according to claim 1, wherein said means includes a chromogenic substrate of peroxidase and a hydroperoxide.
5. A claim according to claim 1, where said substrate is a solid support.
6. A claim according to claim 2 where said chromogenic substrate is on a solid support.
7. A claim according to claim 3 where said hydroperoxide is on a solid support.
8. A claim according to claim 4 where said hydroperoxide and said chromogenic substrate are on a solid support.
9. A claim according to claim 4 where said solid support is a bibulous mat.
10. A claim according to claim 5 where said solid support is a bibulous mat.
11. A claim according to claim 6 where said solid support is a bibulous mat.
12. A claim according to claim 7 where said solid support is a bibulous mat.

13. A claim according to claim 2 where the chromogenic substrate of peroxidase is guaiac.

14. A claim according to claim 2 where the chromogenic substrate of peroxidase is bilirubin.

15. A claim according to claim 2 where the chromogenic substrate of peroxidase is starch and a soluble iodide salt.

16. A claim according to claim 2 where the chromogenic substrate of peroxidase is orthotolidine.

17. A claim according to claim 2 where the chromogenic substrate of peroxidase is p,p'biphenol and a soluble thiocyanate salt.

18. A claim according to claim 2 where the chromogenic substrate of peroxidase is 3,3'5,5'tetramethyl-benzidine and a soluble bromide salt.

19. A claim according to claim 2 where the chromogenic substrate of peroxidase is 3,3',5,5' tetramethyl-benzidine and a soluble iodide salt.

20. A claim according to claim 2 where the chromogenic substrate of peroxidase is orthodianisidine and a soluble iodide salt.

21. A claim according to claim 3 where the said hydroperoxide is a substance which generates a hydroperoxide when moistened.

22. A claim according to claim 3 where said hydroperoxide is an enzyme-substrate system which generates hydrogen peroxide.

23. An indicator according to claim 2 wherein the substrate is a solid support which comprises in different regions of said support antibody against estrogen-induced peroxidase from vaginal fluid, a hydroperoxidase and a chromogenic substrate of peroxide.

24. An indicator according to claim 3 wherein the substrate is a solid support which comprises in different regions of said support antibody against estrogen-peroxidase from vaginal fluid and a hydroperoxide.

25. An indicator according to claim 2 wherein the substrate is a solid support which comprises in different regions of said support antibody against estrogen-induced peroxidase from vaginal fluid and a chromogenic substrate of peroxidase.

26. A method for producing antibodies against estrogen-induced peroxidase by injection of an animal with human vaginal fluid.

27. A method for producing antibodies against estrogen-induced peroxidase using in the lymphocyte hybridoma method human vaginal fluid.

28. A method for producing antibodies against estrogen-induced peroxidase by injection of an animal with an apoenzyme of estrogen-induced peroxide.

29. A method for producing antibodies against estrogen-induced peroxidase using in the lymphocyte hybridoma method an apoenzyme of estrogen-induced peroxidase.

30. A method for producing the apoenzyme of estrogen-induced peroxidase from tissue cultures of estrogen-sensitive tissue.

31. A method for producing the apoenzyme of estrogen-induced peroxidase from recombinant DNA methods.

32. A method for detecting impending ovulation in which a substrate which includes an antibody against estrogen-induced peroxidase is contacted with a vaginal fluid sample and then a test is performed for estrogen-induced peroxidase immunoadsorbed onto said substrate.

33. A method according to claim 32 where said substrate is an inert solid support for said antibody.

34. A method according to claim 33 wherein said test for peroxidase comprises a chromogenic substrate for peroxidase and a hydroperoxide.

INTERNATIONAL SEARCH REPORT

International Application No PCT/US80/00813

I. CLASSIFICATION OF SUBJECT MATTER (If several classification symbols apply, indicate all) [3]

According to International Patent Classification (IPC) or to both National Classification and IPC

INT. CL.[3] A61K 39/00;C12Q 1/66, 1/28; G01N 33/48
U.S. CL. 23/230B; 424/12,85; 435/7,28

II. FIELDS SEARCHED

Minimum Documentation Searched [4]

Classification System	Classification Symbols
U.S.	23/230B; 435/7,28,805; 424/12,85

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [5]

III. DOCUMENTS CONSIDERED TO BE RELEVANT [14]

Category *	Citation of Document, [16] with indication, where appropriate, of the relevant passages [17]	Relevant to Claim No. [18]
X	US,A, 3,817,837, PUBLISHED 18 JUNE 1974, RUBENSTEIN ET AL.	1-34
X	US,A, 4,070,492, PUBLISHED 24 JANUARY 1978, SPECTOR.	1-34
X	US,A, 3,472,738, PUBLISHED 14 OCTOBER 1969, FOSTER.	1-34
X	US,A, 3,644,177, PUBLISHED 22 FEBRUARY 1972, ZYK.	1-34
X	N, CONTRACEPTION, ISSUED JUNE 1975, J.A. BLAIN ET AL, PEROXIDASE IN HUMAN CERVICAL MUCUS DURING THE MENSTRUAL CYCLE, PAGES 677-680.	1-34

* Special categories of cited documents: [15]

"A" document defining the general state of the art
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"T" later document published on or after the international filing date or priority date and not in conflict with the application, but cited to understand the principle or theory underlying the invention
"X" document of particular relevance

IV. CERTIFICATION

Date of the Actual Completion of the International Search [1]: 08 OCTOBER 1980

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